INDUCTION OF IMMUNITY THROUGH THE USE OF *IN VITRO* CELL CULTURES OF MOUSE NEOPLASMS

by

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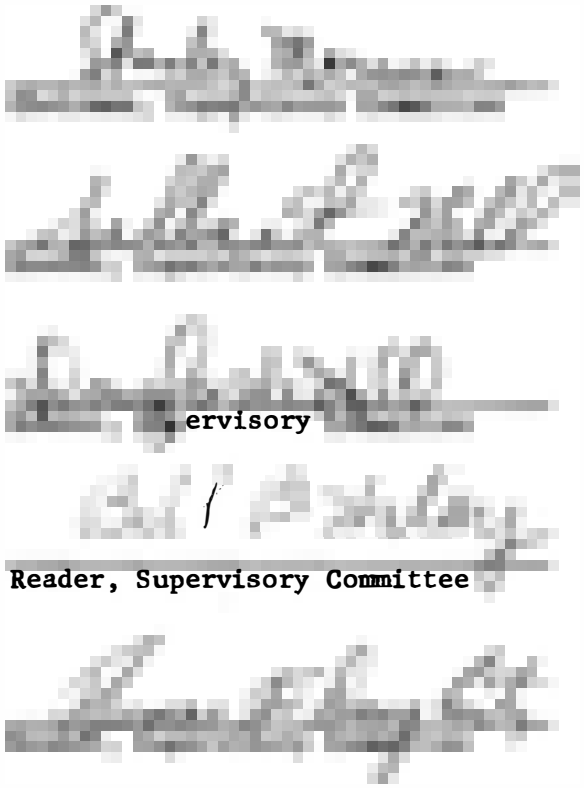

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Reader, Supervisory Committee

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I. INTRODUCTION

In the search for possible clues into the nature of immunological response of a host to cancer, the concept of "self" and "not-self" recognition is encountered. Cancer invades and thrives in a host because that host does not recognize these intruding neoplastic cells as "not-self."

Speculation as to some causes of this lack of recognition might include the idea that in the initial stages these cancer cells may not be antigenic enough to elicit an immunological response in the host, or, if such systems are operable, perhaps the mechanisms are not rapid enough to halt the advance of the neoplasm.

It is noteworthy, however, that apparent spontaneous regressions of cancer may occur. In some cases, hormonal upsets, bacterial or viral invasions have been implicated as possible causes of regression. Whatever the cause, somehow a mechanism must be called into play which activates specific defense mechanisms to enable the host to recognize the invader as "not-self" and allow the body to overcome it.

The search for tumor specific antigens has as yet failed to produce practical results. The failure of such agents as "tissue vaccines", formalin-inactivated and x-irradiated cells as possible immunogens has been attributed to their being non-viable. A need to study viable, slightly altered, immunogenic cells has become more apparent in recent times.

The objectives of the research for this thesis have focused on the use of *in vitro* cultures of tumor cells as a source of altered, but still viable, cells for possible immunogenic agents against the virulent tumor cells of the host. The specific objectives included the following: (a) *in vitro* culture of Ehrlich and S-37 ascites tumors, and other, solid neoplasms of the mouse;

(b) virulence (LD_{50}) determinations for mouse-passage and *in vitro* strains of EAT and S-37; (c) immunogenicity studies with these tumors; and (d) retained virulence and immunogenicity studies of refrigerated S-37 tumor cells.

II. REVIEW OF LITERATURE

A. HISTORICAL DEVELOPMENT OF TISSUE CULTURE TECHNIQUES

The basic idea of eventually being able to culture tissues outside the body had occurred to individuals, particularly embryologists, prior to 1900. However, the first actual cultivation showing unequivocal continuation of function in vitro and offering a reproducible technique must be credited to Harrison (1907). While studying the embryonic development of nervous tissue of the frog, Harrison developed what was to become the "hanging drop" technique of tissue culture. Pieces of tissue were dissected and placed on a coverslip in a drop of lymph which coagulated to secure the tissue onto the coverslip. The preparation was then inverted over a hollow slide and the rim of the coverslip sealed with paraffin. Tissues thus prepared usually continued to proliferate in amoeboid outgrowths of protoplasm from the original transplant, thus forming long nerve fibers. The cultures remained viable from one to four weeks.

Burrows (1910), working under Harrison, improved upon the method by using plasma clots in place of lymph and adapted the method to chick embryonic tissue. Later, Carrel and Burrows (1911a) succeeded in culturing adult and embryonic mammalian tissues, not only in hanging drop cultures but also on large rectangular plates.

One major drawback to the hanging drop method was liquefaction of the clot due to consumption of nutrients and accumulation of waste products and the consequent need for frequent subculturing of fragments to new coverslips. In efforts to overcome these difficulties and to alleviate problems encountered because of frequently disturbing the cultures and possible introducing bacterial contamination, Carrel (1923) developed a flask technique for tissue culture

utilizing flat, round flasks with narrow oblique necks inclined at a 45-degree angle. Modifications included such features as a top opening, two necks, or thin mica plate inserts which facilitated handling or provided means for removing parts for staining, microscopic examination, etc. Both solid and fluid media were utilized for these cultures; that is, fragments of tissue were explanted into a mixture of appropriate plasma and tissue extract, allowed to clot, and then covered with fluid medium. To maintain the cultures, the fluid medium was merely renewed at two, three, or four day intervals. This procedure could be carried out at the rate of about sixty flasks per hour in marked contrast to the slow, tedious hanging drop method of complete subculture at every change in medium.

Maximow's "flying coverslip" method (Maximow, 1925) involved placing the clot on a circular coverslip which was in turn attached to a larger coverslip, inverting both and sealing them over a depression slide. This allowed sterile transfer of the entire culture without removing it from the coverslip.

The roller tube technique of Gey (1933) represented attempts to simplify the work of the tissue culturist still further. Round or hexagonal tubes were fitted into a revolving drum apparatus in such a way that the slow revolution of the drum continually bathed the growing cells in the nutrients of the fluid medium and gases in the tubes. Advantages over previous methods included (a) greater surface area for growth, (b) controlled intervals of contact of cells with media and gases, (c) recovery in the supernate of cells previously lost in liquefaction, (d) more efficient use of media, and (e) less time involved in manipulation of cultures. The cultures lasted several weeks to several months.

Parker (1936) reported success in having cultured breast muscle from chick embryo in single flasks for over a year. This achievement represented

to that date the longest time for animal cells to remain in culture without being transferred. It also demonstrated that a plasma coagulum was not a necessity for prolonged cultivation. One set of cultures did employ a coagulum of adult fowl plasma in Tyrode's solution, but slightly better results were obtained using a fluid medium of adult fowl serum in Tyrode's solution.

An innovation in the site of attachment of cells in culture was next introduced by Evans and Earle (1947). Freshly explanted chick heart fibroblasts and sarcoma cells were seen to proliferate luxuriantly on the undersurface of perforated cellophane. Growth on the glass surface alone was extremely scanty in comparison. The cellophane, as well as the previously used plasma clot matrix, probably served as a physical or mechanical means of adhesion, but the cellophane had significant advantages. For example, the cellophane was chemically inert, transparent, and easy to manipulate, but the plasma clot was chemically undefined, obscured observations due to its bulk, liquefied and destroyed cells, and thus interfered with metabolism, weight, and growth studies. Thus this cellophane method overcame some of the disadvantages of former methods.

The development of single-cell isolation techniques for L-cells by Sanford, Earle and Likely (1948) was the basis for establishment of pure cell lines. Gey's development of HeLa cells (Gey, Coffman, and Kubicek, 1952), Chang's human conjunctiva and liver cells (Chang, 1954), Eagle's KB cells (Eagle, 1955b), the Detroit lines (Stulberg and Berman, 1958), Foley's Sarcoma 180 (Foley and Droplet, 1956), and others followed.

These advances represented a major technological development in the utilization of tissue culture methods for nutritional and physiological studies. For example, using the pure cell line of L-cells and the perforated cellophane method for obtaining massive numbers of cells, Evans *et al.* (1951) prepared

replicate tissue cultures with such ease and accuracy that at least one-hundred replicate cultures could be made by two operators in four hours with an experimental error of less than 5%, 95% of the time.

The demand for a method of tissue culture which would facilitate direct counting of cell populations and overcome the difficulties of measuring growth of compact colonies stimulated Owens, Gey and Gey (1954) to develop a method of culturing cells in suspension. "Tumble tubes" rotated end over end and kept in constant motion prevented cells from adhering to the glass surface. Such cultures could be maintained for forty to fifty days with two-day transfers. Earle et al. (1954a, 1954b) established growth of L-cells in rapid proliferation in fluid suspension using roller tubes rotated at speeds exceeding 10 r.p.m., and also in Erlenmeyer flasks kept in constant motion on a shaker. Many other workers adapted this method to their needs (Siminovitch et al., 1957; Kuchler and Merchant, 1958; McLimans et al., 1957; and Wallace and Cox, 1959).

With the ever-increasing demand for reproducibility of experimental results and quantitation of growth factors has come the increased need for chemically defined media. The early work was carried out using natural animal products almost exclusively, particularly plasma, sera, and embryo extracts. Of course, much work today still employs these reagents. Earliest attempts to try to analyze the requirements to synthesize artificial media began as early as 1911 with Lewis and Lewis and 1913 with Carrel. Healy (1958) has critically documented the development of synthetic media beginning with these works up to the present. The list of chemically defined media in use today includes the following: Medium 199 (Morgan, Morton and Parker, 1950), Medium 858 (Healy, Fisher and Parker, 1955), Eagle's medium (Eagle, 1955a), NCTC 109 (Evans et al., 1956a, 1956b), CMRL 1066 (Parker, Castor and McCulloch, 1957), and Scherer's

medium (Scherer, 1953). This list could be extended, but these media are representative of the group. They contain a mixture of thirty to sixty components including essential amino acids, vitamins, coenzymes, glucose and salts.

Despite the enormous amount of effort that has been expended by the foregoing and other workers (Pumper, 1958; Neuman and Tytell, 1960) to eliminate completely the use of serum in tissue culture and achieve a completely synthetic medium of chemically-defined ingredients, the semi-synthetic medium appears to be the most practical and most widely used for sustaining cultures over a long period of time. A balanced salt solution alone may suffice for mere maintenance for a very brief period, but for extended survival, amino acids, vitamins, co-enzymes and serum protein are required. A synthetic mixture can provide all except serum protein. Thus the semi-synthetic medium of chemically-defined mixtures, balanced salt solution and serum supplements is usually preferred.

Finally it must be noted that no universal medium has yet been found, nor is it likely ever to be found. Source of the cells to be cultured, their metabolic requirements, the intended usage and other factors all will influence the choice of medium and methods of culture to be applied in a given situation.

B. CULTURES OF HUMAN AND ANIMAL NEOPLASMS

Early efforts in tissue culture naturally had as their goal the effecting of the cultivation of the cells *per se*. Investigations into the variations in cytology and nutrition of cells of both adult and embryonic tissue occurred in natural sequence as procedures were developed and improved. Both animal and human tissues were objects of investigation, and studies of malignant as well as normal tissues took place simultaneously in many cases.

Lambert and Hanes (1911a) utilized Burrow's modification of Harrison's hanging drop method to characterize the growth of rat and mouse sarcomata and mouse carcinomata. The growing cells showed chiefly two morphological patterns: (a) long, spindle-shaped cells with thread-like extensions terminating in small prickles, with a central, ovoid or fusiform nucleus, and (b) amoeboid cells with elongated pseudopods terminating in thread-like processes. These same descriptions apply to present-day cell cultures.

Carrel and Burrows (1911a, 1911b) reported culturing both normal and malignant tissues of dog, cat, chicken, rat, and guinea pig in plasma cultures in which homologous plasma alone provided the medium for growth. Some cultures lasted from five to twenty days. Four hundred cultures of Rous sarcoma grew rapidly, showing up to forty times the original number of cells in twenty-four hours, but unfortunately cultures usually degenerated by forty-eight hours. Human carcinoma and sarcoma tissues were harder to cultivate due to liquefaction of the plasma clot. In efforts to overcome this problem, while attempting to culture human connective tissue, Losee and Ebeling (1914) found that diluting the plasma with an equal part of Ringer solution and adding human tissue extract enabled them to keep one culture alive for more than two months, though subcultures still had to be made at one- to four-day intervals.

Hanging drop preparations of fifty-two cultures of spontaneous mouse tumors were made by Lewis and Strong (1934). In contrast to Carrel and Burrow's use of homologous plasma (1911a), these authors found that chicken plasma alone was preferred over mouse plasma or a mixture of chicken and mouse plasma. Also, Grand, Chambers, and Cameron (1935) successfully cultured both human and mouse malignant melanomas for several weeks using Maximow's flying coverslip method

(Maximow, 1925) with human serum for human melanoma and rat serum for mouse melanoma along with chicken plasma and chick embryo extract. An interesting innovation in Grand's work was the addition of bits of normal tissue to maintain the pH of the media made excessively alkaline by growing mouse (but not human) melanomata.

So successful was the work of Gey and Gey (1936) in cultivating numerous mesoblastic tumors, sarcomas, and normal tissues that a summary of their findings is quoted: "The possibility of maintaining by continuous culture large quantities of human and animal malignant and normal cells for various experimental purposes is no more a matter for conjecture but has actually been proved." Gey cultured 332 tissues including fibroblasts, giant cell tumors, fibrosarcomas, spindle-cell sarcomas, etc., and successfully maintained them in culture for periods of time from 54 to 1583 days using slide and/or roller tube cultures with varying proportions of balanced salt solution, bovine and mouse embryo extract, placental cord serum and human or chicken serum. Variations in the medium of a given strain could be made without harming the cells. The same medium could be used for human and animal cells, and malignant and normal cells. He noted that human tumors were as easy (or as hard) to maintain as animal tumors.

Fischer and Davidsohn (1939) combined the techniques of hanging drop coverslip cultures and flask cultures, and thereby succeeded in maintaining a pure strain of carcinoma cells <u>in vitro</u> for over twelve years. The original five small fragments of mouse adenocarcinoma had been subcultured to some 100,000 cultures over the twelve-year period.

Coman (1942) combined the use of solid media with fluid media in roller tube cultures of sarcomas, carcinomas, and various benign growths. Where Coman was interested in studying the subsequent cellular growth and its cytology,

Royle (1946) patterned her work after Coman but studied the stroma and parenchyma of some twenty human tumors. Roller tube cultures were initiated with a chicken plasma-chick embryo extract clot to which a medium of physiological saline and fetal cord serum was added. Subcultures to hanging drop slides utilized a similar medium.

Maximow's coverslip technique was again the method of choice for Murray and Stout (1946) when they cultured biopsy material from eight patients with sympathicoblastoma. The extremely rapid growth and accompanying necrosis of the neurites in tissue culture served as a diagnostic tool in differentiating this tumor from lymphosarcoma (with which it is easily confused clinically). Similar work by these same authors employed tissue culture for determining the histogenesis of mesotheliomas (Stout and Murray, 1942) and schwannomas--specific nerve sheath tumors (Murray, Stout, and Bradley, 1940). Southam and Goettler (1953) also used in vitro cultures in differentiating human epidermoid carcinoma cells from adjacent normal epithelium.

In cytological studies Marcuse (1955) cultured six-hundred consecutive human surgical specimens (including malignant and benign tumors and normal tissues) and achieved 57.5% successful short-term, hanging drop cultures. This figure is more impressive when it is noted that these were consecutive specimens and thus included many not suitable for in vitro culture due to infection, radiation, or use of antiseptics. When used in conjunction with standard histologic procedures of tissue sections and exfoliative cytology, tissue culture preparations thus become a helpful diagnostic tool.

The mid-1950's also saw a great surge of activity in establishing stable cell lines (as cited in the previous section), especially for virus propagation studies. It will be noted that as many or more of these cell lines were of

neoplastic rather than normal origin. Examples are HeLa--epidermoid carcinoma of the cervix, Detroit 6--sternal bone marrow of a patient with carcinoma of the lung, KB--human epidermoid carcinoma.

Stulberg and Berman (1958) in developing the Detroit lines concurrently investigated some twenty human cell lines including malignant, benign, and normal cells. These studies involved explanting suspensions of bone marrow cells, ascitic and pleural fluids, leukocytes, and cells from solid tissues and tumors. The resulting cell lines were used in studies of morphological transformations in vitro, transplantation, chromosomal characterization, and viral susceptibilities. Also, these workers developed a slow freeze process for extended storage and recovery of cells to attempt to alleviate problems encountered with changes in morphology occurring when cells are maintained in tissue culture over long periods of time.

From primary cultures of 142 specimens of normal and neoplastic tissues from animals and humans during a two and one-half year study, fifty-one new cell lines were "established in vitro" (carried as a stock culture for six months) by Foley et al. (1960). Greater success (55%) was achieved with non-neoplastic than with neoplastic tissue (33%). Attempts to culture human sarcoma, bone marrow and peripheral blood of children with acute leukemia, and human lymphosarcomas were unsuccessful. Most interesting was the fact that the medium used for all the cell lines was Eagle's basal medium (Eagle, 1955a) supplemented with 10% whole, pooled human serum for human cell lines and 10% calf or horse serum for animal lines.

Examples of successful cultures of human and animal neoplasms could be compounded indefinitely. Suffice it to say that the materials and procedures are well documented and well established, and they do play a very vital role in many areas of research.

C. CULTURES OF EHRLICH ASCITES TUMOR CELLS

The Ehrlich tumor is by definition (Stewart *et al.*, 1959) "an undifferentiated tumor that originated spontaneously as a carcinoma of the mammary gland of a stock mouse." According to Klein (1950), the ascites variant was developed by Loewenthal and Jahn in 1932 by selective serial transplantation of ascitic fluid of mice inoculated intraperitoneally with suspensions of Ehrlich tumor cells. The name Ehrlich ascites tumor cells, EAT, was then given to the resulting tumor.

Hull (1953) was the first to describe *in vitro* culture of EAT. Using a natural medium containing 10% chick embryo extract, 40% horse serum and 50% Earle's balanced salt solution, Hull maintained Carrel flask cultures of EAT for over eight months. Using quite a similar medium--50% ox serum ultrafiltrate, 10% chick embryo extract, and 40% Hank's balanced salt solution--Siegel (1954) flask-cultured EAT for six weeks in virus-host cell studies. Stained coverslips showed that the cells retained their usual appearance the first week, then later evidenced progressive degeneration, increasing cytoplasmic vacuolation, and karyoplasmic fragmentation. However, many normal cells were still apparent at six weeks, the end of the experimental period.

Powell (1957) utilized short-term double-coverslip cultures employing chick embryo extract, fowl ascitic fluid and fowl plasma in cytological studies. He noted that cell viability and activity was directly proportional to cell density up to the point of overcrowding. He also noted that aggregations of cells were mutually protected by their own diffusion of soluble cell products into the medium, but that isolated cells degenerated due to the loss of this protective substance.

An elaborate mechanical cytogenerator provided the means for Graff and McCarty (1957) to cultivate EAT *in vitro*. This method of sustained tissue culture permitted continuous introduction of nutrients, gaseous exchange, and removal of waste products. The basic medium used here was a modification of Eagle's medium with no serum supplements.

Seeking to learn survival conditions of EAT as a basis for subsequent virus-host cell studies, Eaton and Scala (1958) performed extensive experiments on the nutritional and respiratory activities of short-term cultures of EAT using Hank's balanced salt solution with combinations of glutamine, pyruvate, glutamate, lactate, and glucose. They found that unknown factors in bovine albumin and horse serum did extend survival but amino acids, vitamins, etc., did not do so under the given experimental conditions.

Likewise, Deschner and Allen (1960) achieved an 80-day cultivation of EAT on the surface of glass bottles using 40% Puck's nutrient solution, 45% Hank's balanced salt solution and 15% horse serum. They preferred horse serum to calf serum and chick embryo extract.

A cell line of EAT cells which had been in culture for over two years when reported (Foley *et al.*, 1960) was one of the 51 cell lines so cultured, as reviewed in the previous section. All the animal cell lines established by these workers utilized a medium of 90% Eagle's medium supplemented with 10% calf serum. In contrast to Deschner and Allen, Foley's EAT cells would not survive on horse serum. The work of these two groups represented the first successful cultivations of EAT in static culture on glass to utilize relatively chemically defined media.

In phagocytosis studies employing EAT, Gordon and King (1960) maintained the cells for six months in a medium made by doubling the glucose, amino acid,

and vitamin contents of Eagle's medium. No other metabolites were added.

Jackson, Guiffre, and Perlman (1960) reported growing EAT in suspension as well as static culture. Growth was maintained for over a ten-month period by combinations of static culture in Eagle's or Ziegler's medium supplemented with 10% calf serum and suspension cultures in Waymouth's medium supplemented with 10% calf serum and 0.03% carboxymethylcellulose.

Two other groups achieved successful static cultures on glass. Cailleau and Costa (1961) obtained best results in maintaining EAT in culture for more than one year by using a medium of 70% Medium 199, 12 - 15% fetal calf serum and 15 - 18% Tyrode's balanced salt solution, whereas Ely and Gray (1960) utilized a modification of Medium 858 supplemented with 10% calf serum or horse serum in cultures extending over an eight-month period.

Yet another long-term culture of EAT was reported by DiPaolo (1962) who cultured the cells in static culture on glass for over a year in a medium consisting of 60% Earle's balanced salt solution containing 0.65% lactalbumin hydrolysate, 20% fetal calf serum, and 20% bovine amniotic fluid. Deleterious effects occurred when substitutions or changes in amounts of media constituents were attempted. Calf or horse serum in place of fetal calf serum or 2 - 40% human ascitic fluid in combination with fetal calf, calf or horse serum in Earle's would not support growth. Eagle's medium or Medium 199 likewise failed.

Thus, just as in the case of development of tissue culture techniques and of human and animal neoplasms in general, the <u>in vitro</u> culture of EAT shows much variation in techniques and media employed, depending upon the circumstances under which the work is done and by whom it is done. A general trend toward refinement of techniques and simplification of media can be observed, yet no one definite pattern suffices for all cases.

D. IMMUNOLOGICAL ASPECTS OF TISSUE CULTURE

Many of the problems encountered in attempts to immunize a host against his own tumor involve the concept of "self" and "not-self" recognition. Cancer and other neoplasms invade and thrive in a host because the body does not recognize these intruders as "not-self." Subtle antigenic differences between normal and neoplastic cells may exist. Apparently, however, in the initial stages, the neoplastic cells may not be antigenic enough to elicit an immunological response in the host, or if such systems are operable, perhaps the mechanisms are not rapid enough to halt the advance of the neoplasm. Yet apparent spontaneous regressions can occur. In some cases hormonal upsets, bacterial or viral invasions have been implicated as causes of regression. Somehow, a mechanism must be called into play which activates specific defense mechanisms to allow the body to overcome the invader.

The rationale underlying the immunological approach to neoplastic disease implies that specific antigenic components must exist in neoplastic cells but be absent in normal cells, and that specific immune reactions could be utilized to distinguish this antigenic difference (Hirsch, 1959). Proof of the actual existence of such antigens and their accompanying antibodies has been the object of research endeavors for the past several decades, but as yet no conclusive proof of the reality of such entities has been forthcoming.

Evidence for the presence of tumor-specific antigens in transplanted, carcinogen-induced and spontaneous tumors is cited by Hirsch (1962). The classical method of demonstrating tumor immunity by immunizing with tumor cells and then challenging with the same tumor cells is usually carried out with transplante tumors. Any immunity resulting from such procedures has often been considered to be due to the presence of tumor-specific antigens. The likelihood that

actually a homograft or heterograft response occurs and that the immunity is not against the tumor tissue per se but rather against antigens common to the tumor and normal tissue of the animal in which the tumor originated has been stressed by Prehn and Main (1957). Hirsch (1962) likewise warns of the pitfalls of extrapolating the results of work with transplanted tumors to apply to work with spontaneous tumors.

Evidence that carcinogen-induced tumors may bear true tumor-specific antigens is stronger than corresponding evidence for transplanted or spontaneous tumors, as is shown in work by Foley (1953), Prehn and Main (1957) and Gross (1943). "Antigen X" and its corresponding antibody have been described by Amos and Day (1957), and Gorer (1956). Also, McKenna, Sanderson, and Blakemore (1962) have described extracting distinctive antigens from HeLa and J-111 cells by treatment with fluorocarbon.

Two current theories of origin of cancer, the virus theory and the somatic mutation theory, are closely related to tumor-specific antigens (Hirsch, 1962). Specific antigens of viruses could be considered as tumor-specific antigens against which immunotherapy could be directed. Somatic mutation may result in an altered protein or other macromolecular molecule that would serve as the antigen. Burnet (1959) believed that "somatic mutation is basically responsible for all forms of cancer and that genetic, chemical, and viral influences merely provide the conditions which can accelerate the emergence of the effect of somatic mutation." If such is the case and a specific antigen is involved, the role of immunotherapy in treatment of cancer could be very significant.

In the absence of isolation of unique antigen and antibody, a number of clinical treatments of cancer patients in the past have involved the use of cancer tissue itself, usually altered in some way, as the hypothetical cancer antigen.

In a review citing the work of at least twenty investigators, Southam (1961) describes various pretreatments of the cancerous tissue. Autogenous vaccines to various tumors were made by (a) formalinizing, (b) phenolizing, (c) homogenizing in alcohol, drying and resuspending in saline, and (d) extracting in alcohol, (e) X-irradiating, (f) chemically extracting "globulins," and (g) freezing. Though some patients showed improvement and some responded to combinations of vaccines and surgery or serum therapy, few actual cures were claimed to have been effected by these treatments.

In recent studies on establishment of cancer resistance in mice, Larson, Slater, and McKee (1961) noted that metabolizing or partially viable cells were apparently required for production of resistance, as cells which had been X-irradiated with medium strength exposure were more effective in conferring resistance than those with high dosage X-irradiation. Likewise, Donaldson and North (1960) obtained better results with moderate than with high amounts of nitrogen mustard treatment of cells. Similarly, Hill and Marcus (1960) demonstrated that formalin-killed EAT failed to induce immunity whereas iv injections of sublethal doses of live cells did increase resistance to subsequent ip challenge with lethal doses of live cells. Perhaps some of the failures of the "tissue vaccines" previously cited were due in part to use of non-viable cells.

Could tissue culture of neoplastic tissues possibly supply a source of viable cells which upon injection into the host might elicit an immunological response in the host? The hypothesis has been advanced that growth in tissue culture could alter the virulence mechanisms of tumor cells without harming viability. The following review of findings reveals some of the contrasting views on the subject.

For cells in general, three cases of lowered virulence of cells in tissue

culture are cited. Lambert and Hanes (1911b) reported that sarcoma cells grown in plasma of immune animals and in plasma of normal animals when inoculated back in vivo showed the same results, but when these two in vitro groups were compared with implants of tumor tissue directly from animals, the percentage of takes was significantly less and the number of regressions greater in the in vitro implants The authors attributed their results to the lowered virulence of their tissue-cultured cells. The studies of Gey, Bang and Gey (1954) on prolonged tissue culture of cells reported loss of transplantability of Earle's strain L mouse fibroblasts, his own strain of rat fibroblasts, and deBryn's MB-III strain of lymphoblastic cells of tumorous origin. Also, the American Type Culture Collection Registry of Animal Cell Lines for 1964 indicates that establishment of a second cell line of Sarcoma 180-mouse was necessitated due to the loss of tumorigenicity of the earlier line (Foley and Droplet, 1956) with increased time in culture.

Ability of cells in tissue culture to retain their tumor-producing ability has been reported by several investigators. Hotchin's Novikoff rat hepatoma cells produced tumors upon intraperitoneal inoculation into adult rats after eight weeks in tissue culture (Hotchin, 1957). Fischer and Davidsohn's strain of carcinoma cells (1939) retained its malignant properties over its twelve-year in vitro culture period. Walker 256 tumor cells cultured by Tuttle and Foushee (1961) still induced tumor formation after eighteen months in cultivation.

In contrast, the tendency for normal cells to become malignant with an increased time in culture has also been observed. Moore, Southam, and Sternberg (1956) reported that four lines of Chang normal liver, kidney, and conjunctiva cells after more than fifty passages in tissue culture developed cytological and chromosomal changes and produced tumors in specially treated rats and human volunteers. Similarly, Westwood, MacPherson and Titmus (1957) working with

eighteen cell lines obtained from normal tissues of rabbits, guinea pigs, monkeys and human embryo noted that six lines transformed to exhibit in vitro characteristics of malignant cells, but did not produce tumors upon injection into homologous and heterologous hosts.

Production of malignancy in vitro was simulated by Earle and Nettleship (Earle, 1943; Earle and Nettleship, 1943) by subjecting C_3H mouse fibroblast cultures to the action of methylcholanthrene in vitro. Cells from such cultures produced malignancy when reinjected into susceptible mice.

With respect to Ehrlich ascites tumor cells, Deschner and Allen (1960) and Jackson et al. (1960) reported a decrease in virulence with increased time in culture, whereas Foley et al. (1960), DiPaolo (1962), and Graff and McCarty (1957) indicated no significant loss in virulence.

Cailleau and Costa's work with Ehrlich ascites tumor cells (1961) compares the virulence of tissue culture cells with the corresponding wild mouse-passage cells. Their most interesting findings indicate that a decrease in virulence did occur; 10^5 or more tissue culture cells were required to produce a high per cent of tumors, compared with 10^3 mouse-passage cells. Concomitant with this loss of virulence, Cailleau and Costa detected an increased ability of the tissue culture cells to induce immunity. Mice given sublethal doses of 10^3 tissue culture cells all survived subsequent injection with the lethal 10^5 dose of tissue culture cells. Likewise, this sublethal dose of 10^3 tissue culture cells partially protected against challenge with lethal 10^3 doses of mouse-passage cells.

In extensive histological studies, Baillif (1960, 1964) has followed the cytological changes occurring in reticuloendothelial tissues of mice injected and immunized with EAT. The responses were interpreted as defensive mechanisms and were reflected in the reticuloendothelial cell derivatives of lymph nodes, spleen, and possibly adrenal cortex. The pattern of response differed greatly, depending on the success or failure of the anti-tumor reaction.

III. MATERIALS AND METHODS

A. TUMORS

1. Ehrlich Ascites Tumor (EAT).

Two strains of EAT cells were utilized. The first was obtained from the Sloan-Kettering Institute prior to 1958, and has been maintained by serial propagation in mice through weekly transfers of tumor cell-containing ascitic fluid. Its modal chromosome number is 60 with a range from 41-83 (Oshiro, 1963). This strain served as a source of cells throughout the course of the research described.

The second strain of EAT was used in only a limited number of the experiments since only tissue culture cells (no corresponding mouse passage cells) were available. Subcultures of the ESS strain of Cailleau and Costa (1961) were acquired in 1961, after over two years of tissue culture passage by those workers. The chromosome numbers for this strain range from 54-71, with 65 as the modal value.

2. Sarcoma-37 Ascites Tumor (S-37).

The S-37 tumor cells used in this research were derived from serial propagation, by weekly transfer of tumor cell-containing mouse ascitic fluid, of a tumor strain obtained in 1958 from the National Institute of Health. Like EAT, it originated in the early 1900's as a spontaneous, undifferentiated, solid, mouse mammary gland tumor (Stewart, et al., 1959) from which a transplantable ascites variant was later developed. Neither S-37 nor EAT is strain-specific for mice.

3. C_3H_f Mouse Mammary Carcinoma.

In an inbred strain of C_3H_f mice maintained by the Department of Surgery, University of Utah, some of the breeding female mice spontaneously develop carcinoma of the mammary glands at about one year in age.

Week-old, lateral, subcutaneous transplants of one such spontaneous tumor into six male C_3Hf mice were obtained for the studies to be reported.

4. Murine Leukemia Tumor.

Transplants of a murine leukemia tumor carried in CBA mice in both intact and ascites form were obtained from the Department of Anatomy, University of Utah.

B. MICE

For the experiments involving EAT and S-37 tumors which are not strain-specific, random-bred albino mice (Mus musculus) of mixed sex were used. The mice were caged in 7 x 13 inch plastic cages in random groups of 10 or less. They received a diet of Purina laboratory chow and water ad libitum.

For the strain-specific tumors, tumor-bearing mice of the respective inbred C_3Hf and CBA lines were used.

C. MEDIA COMPONENTS

In general, the media used for tissue cultures consisted of four components: (a) a chemically-defined basal medium, (b) balanced salt solution, (BSS), (c) a serum supplement, and (d) antibiotics. Variations of each of these components in different combinations yielded the individual media for specific experiments.

1. Chemically-defined Basal Media.

A number of commercially available, chemically-defined, synthetic media formulae are well-recognized for use in preparing tissue culture media. Their composition includes various amino acids, vitamins, enzymes, co-enzymes, salts and a carbohydrate source as components essential for growth of cells. The exact compositions of those utilized in this research may be found in the respective references.

The following basal media were used:

(a) NCTC 109 (Evans et al., 1956 a, 1956 b)

(b) CMRL 1066 (Parker et al., 1957)

(c) TC 858 (Healy et al., 1955)

(d) TC 858, modified (Ely and Gray, 1960)

(e) TC 199, (Morgan et al, 1950)

(f) MEM, Eagle (Eagle, 1955 a)

Two additional sources for basal media were used:

(a) a 5% solution of lactalbumin hydrolysate (LAH) in Hanks' BSS prepared in the laboratory

(b) a dialysate of yeast extract (DYE) prepared by Dr. Yoshio Aoki, Dept. of Microbiology, University of Utah, and used with Hanks' BSS.

The basal media were all stored at refrigeration temperatures in sterile, 10X or 1X concentrated solutions.

2. Balanced Salt Solutions.

Balanced salt solutions (BSS) were prepared according to the formulations of Hanks, Earle, and Tyrode. All were prepared in 10X concentration, sterilized by sintered glass filtration and stored at room temperature.

3. Serum Supplements.

Sterile calf serum was prepared in the laboratory from local slaughterhouse sources. Fetal calf serum was obtained from a commercial source (Colorado Serum Company). Lamb serum, mouse and human ascitic fluid and chick embryo extract, remaining after previous experiments in this laboratory, were utilized in a limited number of experiments to be reported. All serum supplements were stored at -70° C.

4. Antibiotics.

Penicillin G at 100 units/ml and Streptomycin at 100 ugm/ml were

added to all media. In addition some media received Fungizone (Amphotercin B) at 2 ugm/ml for control of possible fungal contamination.

5. pH Indicator.

All media contained phenol red as a pH indicator. The final pH after all components were combined was adjusted to 7.0 ± .2 by dropwise addition of sterile 4.4% sodium bicarbonate solution.

D. CULTURE VESSELS

All cultures were designed to achieve static culture on the surface of glass tubes and bottles. The following vessels were used: (1) 16 x 150 mm Leighton tubes with rubber-lined screw caps, (2) 16 x 75 mm Leighton tubes with plastic corks, (3) 250 ml and 160 ml rectangular milk dilution bottles (Pyrex) with rubber-lined screw caps, (4) 1000 ml Blake-type rectangular bottles fitted with rubber corks.

All glassware was boiled for 30 minutes or autoclaved in (Haemosal-type) dishwashing compound and rinsed well with tap water. This was followed by machine or hand washing, rinsing, and distilled water rinse prior to final sterilization by autoclaving.

E. PREPARATION OF CULTURES

1. Ascites Tumors.

Two procedures for preparation of cultures of ascites tumors were used. (a) Approximately 5-8 ml. of tumor cell-containing ascitic fluid was aseptically extracted from two to four mice, pooled, and diluted with BSS. The resulting mixture was centrifuged lightly (500 rpm for 5 min.) and supernate discarded. The precipitated cells were washed three times with fresh BSS. An aliquot of the resuspended cells was stained with 0.5% eosin, counted in a Spencer Bright-Line counting chamber, and then diluted to the desired amount for inoculation into the media in culture

tubes and bottles. (b) The tumor cell-containing mouse ascitic fluid was diluted with BSS, centrifuged lightly, and the supernate discarded. The packed cells were then suspended in 1 ml. of fetal calf serum medium, layered over the flat surface of Leighton tubes, and allowed to stand ten minutes to permit a coating of the glass with cells. The excess was then decanted and 5 ml. of fresh medium added. This second procedure was applied only to early experiments with EAT.

2. Solid Tumors.

For preparation of cultures from solid tumors experiments were designed (a) to compare results of a trypsinization procedure against merely the mincing and washing of the tumor tissue and (b) to compare a modified plasma clot technique with merely mincing and filtering the tissue.

When tumor size in the mice had reached approximately 1 cm., the mice were sacrificed by exsanguination and the tumorous material aseptically removed and placed in appropriate sterile tissue culture medium or BSS.

Trypsinization was carried out by adding to a portion of the excised, pooled tumor material 10 ml. of 0.5% trypsin at 37°C and stirring the mixture with a magnetic stirrer at slow speed for 10 minutes. The supernate containing loose cells was then decanted into a cold test tube and transferred to the refrigerator in order to halt the action of the enzymes. More trypsin was added to the flask and the procedure repeated four times. The supernates were pooled, centrifuged at medium speed for five minutes, and the sedimented cells then were resuspended in culture medium.

The remainder of the tissue, not trypsinized, was chopped into small pieces, then finely minced and suspended in BSS. The mixture

was centrifuged and washed three times before cells of the sediment were planted into appropriate media in the culture bottles.

For preparing cultures by the plasma clot technique, the tissue was handled as little and as gently as possible in efforts to keep the tissue intact. Scalpel blades were used in scissor fashion to cut 1 to 2 mm squares of tissue which were placed on a small rectangular coverslip in a film of chicken plasma. A tiny drop of chick embryo extract was then added to form the clot. The coverslips were then inserted into the small Leighton tubes and medium was then layered over them.

The remainder of the tissue, this time, was finely minced, bathed in balanced salt solution, and filtered through glass wool. The cells passing through the filter were then cultured.

All cultures were incubated at 37°C in static position, and read microscopically at regular intervals for signs of growth of cells and adherence to the glass surface. Medium was renewed in the tubes based on these observations and as needed to compensate for pH changes due to the metabolic activity of the growing cells.

F. MEDIA AND GLASSWARE CONTROLS

HeLa cells and FL (continuous passage of human amnion) cells from established cell lines in constant and continual use at the Utah State Health Laboratory were obtained. Cultures of these cells prepared in the same manner as for tumor cells served as procedural controls on the glassware, media, materials, and methods for culturing the tumor cells.

G. TRYPSINIZATION

The trypsinization procedure for harvesting cells from the glass surfaces consisted of the following steps: (1) the old medium was drained and

the surface of the monolayer rinsed with sterile 0.9% physiological saline solution (to remove any remaining inhibiting serum in the medium), (2) 0.25% trypsin was layered over the surface and allowed to stand except for occasional agitation until the monolayer "peeled off" grossly and attached cells could no longer be observed microscopically, (3) the suspension of cells was decanted into a centrifuge tube with an equal volume of serum-containing medium (to neutralize the trypsin), and the mixture washed two more times with fresh medium, (4) the sedimented cells were then resuspended, a sample eosin-stained and counted, and dilutions made as needed for tissue culture passage or studies in mice.

H. MOUSE REINOCULATION STUDIES

1. LD_{50} Determinations.

Verification of previously determined LD_{50} values (Oshiro, 1963) for mouse passage cells of EAT and S-37 tumors was carried out as follows: (a) cells were prepared as for cultures of ascites tumors; (b) dilutions were set up to contain, per 0.5 ml volume, graded doses based on numbers of unstained, presumably viable cells , to bracket the former LD_{50} values; (c) groups of ten mice per group were injected with each specified dose for a given route of injection; i.e., S-37 was injected in graded doses via intraperitoneal (ip), subcutaneous (sc), and intravenous (iv) routes, and EAT via ip and sc routes; (d) deaths and incidence of tumors were observed and recorded up to thirty days after injection.

LD_{50} values for EAT cells after six weeks in culture were determined in a similar manner after harvesting cells from culture by trypsinization.

2. Retained Virulence.

Experiments were designed to test whether tumor cells retain their virulence when stored in the cold over an extended length of time. S-37 cells from ascitic fluid of tumorous mice, after being stored in the refrigerator for varying length of time, were eosin-stained for viable cells and inoculated into mice based on total number of cells (stained and unstained). Ten mice per group were injected ip with 10^4 cells from the various refrigerator samples. Another group of ten mice serving as controls received 10^4 fresh mouse passage cells ip, as this dose constitutes an approximate LD_{50} for this route of inoculation. Deaths and incidence of tumors were again recorded up to thirty days.

3. Immunization.

The same sets of refrigerated S-37 cells which were checked for retained virulence were tested for immunogenicity in other groups of mice in the following manner. Groups of ten mice each received, with an 11-day interval between, two ip injections of 10^3 refrigerated cells as sublethal, immunizing doses. Thirty days after the second injection, a challenge dose of 10^5 fresh, unrefrigerated, mouse passage cells were given intraperitoneally. Deaths and incidence of tumors were recorded up to thirty days after the last challenge dose. An additional group of mice receiving mouse passage cells in all three injections served as "immune" controls. Still another group of mice became "normal" or "unprotected" controls by being subjected to only the final challenge dose of mouse passage cells.

A very limited experiment to ascertain immunogenicity of EAT cells after six weeks in culture was carried out in a similar manner (using only ten mice). The immunizing dose was one iv injection of 10^5 EAT cells from *in vitro* culture. The challenge dose of 10^5 mouse passage cells given ip followed after 30 days. The mortality ratio was noted at the end of another 30 days.

IV. RESULTS

A. IN VITRO CULTURES

Initial efforts involved attempts to establish the mouse-passage strain of EAT as a continuous cell line in in vitro culture.

Variations in experimental conditions were designed to explore trends in effects on growth of (a) presence or absence of serum, (b) the use of calf serum versus fetal calf serum, (c) the presence or absence of carbon dioxide during incubation, and (d) variation in size of inoculum. Of these variables, only (a) was demonstrated to effect results. Details of experimental conditions and a summary of observations are shown in Table I.

Fairly conclusive evidence that serum was beneficial under the conditions of these experiments was indicated by the fact that of the first sixteen tubes, the eight which contained no serum failed to support growth; cellular degeneration was apparent within 24-48 hours, and all cells had died within 72 hours. In the eight tubes with serum, healthy cells were seen in all tubes up to 72 hours with cells in some tubes showing signs of growth and division at six days.

Though encouraging signs of beginning growth were observed, especially in some of the cultures employing medium TC 199 with fetal calf serum, no sustained growth of tumor cells was achieved. All attempts ended in degeneration of cells at three to seven days.

The appearance of the cultures initiated with TC 199 plus fetal calf serum prompted further trials with TC 199 in combination with various serum supplements, namely, human ascitic fluid, lamb serum and mouse ascitic fluid. As shown in the top of Table II comparing these results with the previous cultures, the mouse ascitic fluid was least helpful in initiating growth.

Efforts then centered around the tissue culture strain of cells previously

TABLE I

IN VITRO CULTURE OF ERHLICH ASCITES TUMOR CELLS

Date Started	Tube No.	Composition of Medium	Incubation Conditions	No. of Cells in Inoculum	Method Used*	Summary of Daily Microscopic Observations
7-14-61	1a 1b	100% NCTC 109	37°	Undetermined	1	Cells maintained 24-48 hours, degenerated by 72 hours
"	2a 2b	"	37°, CO_2	"	1	Cells maintained 24-48 hours, degenerated by 72 hours
"	3a	90% NCTC 109, 10% CS	37°	"	1	At 6 days, good dividing cells; at 7 days, degenerated and oval shapes only
"	3b	"	37°	"	1	Cells began degenerating third day
"	4a 4b	"	37°, CO_2	"	1	Oval shapes appeared third day; little or no change thereafter
"	5a 5b	100% CMRL 1066	37°	"	1	Cells maintained 24-48 hours, degenerated by 72 hours
"	6a 6b	"	37°, CO_2	"	1	Cells maintained 24-48 hours, degenerated by 72 hours
"	7a	90% CMRL 1066, 10% CS	37°	"	1	Dividing cells seen on fourth day, fewer on fifth, degenerated on sixth day
"	7b	"	37°	"	1	Degenerated by fourth day
"	8a 8b	"	37°, CO_2	"	1	Good growth through third day, degeneration partial or complete by fourth day
7-18-61	9a 9b	90% TC 858, 10% CS	37°	"	1	Seemed to start very well, some signs of elongation, dividing, and adherence, but less by third day and degeneration thereafter
"	10a 10b	"	37°, CO_2	"	1	

TABLE I (Continued)

Date Started	Tube No.	Composition of Medium	Incubation Condition	No. of Cells in Inoculum	Method Used*	Summary of Daily Microscopic Observation
7-26-61	11a	85% TC 858, 15% FCS	37°	20,000	1	Five out of 6 tubes completely overrun by
	11b	"	37°	100,000	1	Staphylococcus contamination at 48 hours;
	11c	"	37°	200,000	1	sixth tube, no evidence of growth. Anti-
"	12a	"	37°, CO_2	20,000	1	biotics had been omitted from this medium.
	12b	"	37°, CO_2	100,000	1	
	12c	"	37°, CO_2	200,000	1	
7-28-61	1a	85% TC 858, 15% FCS	37°	200,000	1	Good healthy cells at 24 hours, some signs
	1b					of division, but little evidence of growth
"	2a	"	37°, CO_2	200,000	1	at 72 hours--degenerating cells only there-
	2b					after
8-2-61	1a	85% TC 199, 15% FCS	37°	500,000	1	At 48 hours**, almost solid layer of cells;
	1b	"	37°, CO_2	500,000	1	some pointed, dividing cells
"	2a	85% TC 199, 15% FCS	37°	200,000	1	At 48 hours, mostly only round cells
"	2b	"	37°, CO_2	200,000	1	At 48 hours, good aggregation, possible division
"	3a	70% TC 199, 15% FCS, 15% EBSS	37°	500,000	1	At 48 hours, spreading and dividing ad-
	3b	"	37°, CO_2	500,000	1	hering cells; spindle-shaped cells
"	4a	70% TC 199, 15% FCS, 15% EBSS	37°	200,000	1	At 48 hours, good healthy cells; some
	4b	"	37°, CO_2	200,000	1	elongating and dividing
8-3-61	5a	85% TC 199, 15% FCS	37°	8.5 x 10^6/ml	2	At 24 hours**, dense, almost solid cover
	5b	"	37°, CO_2	"		of cells; many spindle-shaped, dividing cell
"	6a	70% TC 199, 15% FCS, 15% EBSS	37°	"	2	At 24 hours, solid packed cells, mostly
	6b	"	37°, CO_2	"	2	round, but many pointed, possibly dividing cells
"	7a	70% TC 199, 15% FCS, 15% TBSS	37°	18 x 10^6/ml	2	At 24 hours, cells dying and degenerating
	7b	"	37°, CO_2	"	2	--inoculum was not fresh, 24 hours old

TABLE I (Continued)

Date Started	Tube No.	Composition of Medium	Incubation Conditions	No. of Cells in Inoculum	Method Used*	Summary of Daily Microscopic Observations
8-4-61	7a'	70% TC 199, 15% FCS, 15% TBSS	37^{o}	2.5×10^{6}/ml	2	Almost solid cover on initial incubation**
	7b'	"	37^{o}, CO_2	"	2	

*See text, Preparation of cultures, p. 23.
**Observations up to and including 8-4-61 only.

For media number designations, see text, Media, p. 22.

Others: CS - calf serum
FCS - fetal calf serum
EBSS - Earle's balanced salt solution
TBSS - Tyrode's balanced salt solution

TABLE II

IN VITRO CULTURE OF ERHLICH ASCITES TUMOR CELLS

No. of Cultures	Medium*	Serum Supplement	(Days) Survival Time
	Mouse-passage strain		
4	100% NCTC 109	None	2
4	90% NCTC 109	10% calf serum	6
4	100% CMRL 1066	None	2
4	90% CMRL 1066	10% calf serum	5
4	90% TC 858	10% calf serum	3
10	85% TC 858	15% fetal calf serum	3
6	85% TC 199	15% fetal calf serum	6
4	70% TC 199, 15% Tyrode's BSS	15% fetal calf serum	4
6	70% TC 199, 15% Earle's BSS	15% fetal calf serum	4
1	70% TC 199, 15% Tyrode's BSS	15% human ascitic fluid	5
1	70% TC 199, 15% Tyrode's BSS	15% lamb serum	5
1	70% TC 199, 15% Tyrode's BSS	15% mouse ascitic fluid	2
	Cailleau and Costa's strain		
	70% TC 199, 15% Tyrode's BSS	15% fetal calf serum	Over one year
	70% TC 199, 15% Tyrode's BSS	15% human cord serum	Over six weeks
	90% TC 858 modified	10% fetal calf serum	1
	90% NCTC 109	10% fetal calf serum	1
	90% CMRL 1066	10% fetal calf serum	1

*For media number designations, see text, Media, p. 22.

described. As shown in Table II also, results with this second strain of cells showed that when Cailleau and Costa's choice of medium was used for that strain of cells, good proliferation and continued subcultures of cells were possible for over a year. Making the slight change to human cord serum in place of fetal calf serum likewise seemed acceptable. (No attempt was made to continue to culture the cells in human cord serum for longer than the six weeks observed.) However, the attempts to culture this strain using the other three media failed altogether.

Efforts to harvest the cells from the monolayers of successful subcultures were made (a) by gentle scraping with a sterile loop or rubber policeman, and (b) by the trypsinization procedure previously described. Neither of these procedures, however, yielded suspensions of cells with viable cell counts high enough to be of value in subsequent experiments such as mouse inoculation studies. These methods were sufficient for subcultures for tissue culture passage, but even the largest bottles used for culture (approximately 9 x 25 cm surface area) yielded only about one million viable cells.

One venture was made to inoculate mice with cells harvested from tissue cultures of this strain, but no tumor cell growth was observed in the inoculated animals.

Though work with this strain of EAT was partially successful, i.e., it was the only strain which could be grown in tissue culture, further experiments with these cells were discontinued for three reasons. First, the original corresponding mouse passage strain of the tumor was no longer available. Second, harvest of cells failed to yield sufficiently high numbers of cells for subsequent use. Third, continued time in tissue culture had altered the cells beyond the point of usefullness for proposed immunological studies. This is further considered in the discussion.

Table III summarizes the results of attempts at in vitro culture of the solid spontaneous mammary carcinoma of C_3H_f mice. Work with this tumor was suggested by the hypothesis that spontaneous tumors (or tumors of recent origin with very few transplant generations) might be more readily grown in tissue culture than tumors which had been subjected to extended animal passage.

Again, no long-term cultures of the cells were achieved, but a limited amount of growth activity was observed in some cultures. In the first set of experiments, the only cultures showing growth were those prepared from the minced, washed, non-trypsinized cells cultured in the medium containing 60% Earle's, 30% TC 199, and 10% calf serum. A slow, epithelial-type growth was observed here. In the second set, the finely minced, filtered cells cultured in the same medium offered the most encouraging signs of growth. Both fibroblast and epithelial-type growth appeared.

An experiment involving injection of these cultured cells into the same strain of C_3H_f mice failed to produce tumors in the recipient mice. In this experiment each of 2 mice received approximately 4×10^5 cells.

Similar limited experiments with the solid form of Dougherty's leukemia tumor of CBA mice yielded no successful cultures.

Research effort with ascites tumors was then renewed, with interest focused on the S-37 tumor previously described. Cultures of both S-37 and EAT were initiated using the following media: (1) 90% Hank's BSS, 5% lactalbumin hydrolysate, and 5% calf serum, (2) 60% Hank's BSS, 30% 199, and 10% calf serum, and (3) 90% Minimum Essential Medium, Eagle, with 10% calf serum.

Cultures in the first two media were negative in showing evidence of growth. Both types of tumor cells in the third medium, however, manifested slight but progressive growth. The S-37 cells occurred in compact, slightly spreading

TABLE III

IN VITRO CULTURE OF C_3H_f MAMMARY CARCINOMA

Exp.No.	Media Used*	Preparation of Tissue Prior to Culture	Culture Vessel	Results
1	85% TC 199, 15% FCS	Trypsinized	250 ml. milk dilution bottle	No growth
2	85% TC 199, 15% FCS	Non-trypsinized; minced, washed	250 ml. milk dilution bottle	No growth
3	60% EBSS, 30% LAH, 10% CS	Trypsinized	250 ml. milk dilution bottle	No growth
4	60% EBSS, 30% LAH, 10% CS	Non-trypsinized; minced, washed	250 ml. milk dilution bottle	No growth
5	60% EBSS, 30% TC 199, 10% CS	Trypsinized	250 ml. milk dilution bottle	No growth
6	60% EBSS, 30% TC 199, 10% CS	Non-trypsinized; minced, washed	250 ml. milk dilution bottle	Slow, epithelial-type growth
7	60% EBSS, 30% mixture of NCTC 109 and CMRL 1066, 10% CS	Finely minced, filtered	250 ml. milk dilution bottle	Limited amount of metabolic activity, but some epithelial-type growth
8	60% EBSS, 30% mixture of NCTC 109 and CMRL 1066, 10% CS	Modified plasma clot technique	16 x 75 mm. Leighton tube	No growth
9	60% EBSS, 30% TC 199, 10% CS	Finely minced, filtered	250 ml. milk dilution bottle	Good growth, both fibroblast- and epithelial-type growth
10	60% EBSS, 30% TC 199, 10% CS	Modified plasma clot technique	16 x 75 mm Leighton tube	No growth

*For media number designations, see text, Media, p. 22.

Other abbreviations: FCS - fetal calf serum; CS - calf serum; EBSS - Earle's balanced salt solution; LAH - Lactalbumin hydrolysate

clumps, with possibly twelve to fifteen such clumps per bottle. Most of the cells appeared round, some had large vacuoles, and others were slightly pointed and elongated. The EAT cells were similar in morphology but many of the clumps evolved into hollowed-out "rings" of cells. Possibly aging of the cells in the center of the clumps, lack of nutrients or accumulation of toxic products were responsible for this phenomenon. Slow but progressive growth continued for a short time in cells at the periphery of the clumps.

The S-37 tumor seemed to grow better than EAT in most respects; therefore, a series of culture attempts with S-37 ensued. The first section of Table IV lists the details of these experiments. Efforts were made to replicate and then enhance growth through use of the medium containing 90% MEM, Eagle and 10% calf serum. Also, variations in (a) the number of cells for initiating cultures and (b) the relative concentration of calf serum in the medium were designed to test the effects of increases in these factors.

The results shown in Table IV indicate that Eagle's medium will achieve at least a measure of success in supporting the growth, or at least maintenance, of S-37 tumor cells in vitro. Five of the fourteen experiments listed resulted in apparent attachment to glass, growth, and proliferation of cells. The cells seemed to maintain a fairly uniform appearance, remaining round, though sometimes vacuolated, even when attached to the glass. Almost epithelial-like clusters of cells increased in size and number as cells attached and grew on the glass surface. Very few elongated, pointed, spindle-shaped cells were observed. Almost complete monolayers, though slow in developing, were achieved. Trypsinization for harvesting cells from the most proliferative cultures yielded subcultures of apparently healthy cells (50-60% viable when eosin-stained). The cells of the subcultures, however, would not attach to the glass but rather appeared to proliferate in suspension.

TABLE IV

IN VITRO CULTURE OF S-37 TUMOR CELLS

Date	No. of Cultures	Medium*	No. of Cells x 10^6/Bottle	Results
1-9-64	3	90% MEM, Eagle, 10% CS	Undetermined	Good proliferation for 3½ weeks, then gradual decline
1-9-64	2	90% LAH medium, 10% CS	Undertermined	Nil--less than three days
1-17-64	2	90% MEM, Eagle, 10% CS	1	Maintained one week
1-17-64	2	90% MEM, Eagle, 10% CS	5	Nil--less than three days
1-17-64	2	90% MEM, Eagle, 10% CS	10	Maintained one week
3-25-64	2	90% MEM, Eagle, 10% CS	10	One bottle of each, five to seven days;
3-25-64	2	80% MEM, Eagle, 20% CS	10	other bottle of each, less than three days
3-25-64	2	70% MEM, Eagle, 30% CS	10	Both bottles, five to seven days
4-22-64	1	90% MEM, Eagle, 10% CS	1	Maintained seven to ten days
4-22-64	1	90% MEM, Eagle, 10% CS	1	Used for trial trypsinization at six days
4-22-64	1	80% MEM, Eagle, 20% CS	1	Cloning and proliferation evident at one
4-22-64	1	70% MEM, Eagle, 30% CS	1	month; subcultured
4-22-64	2	90% LAH medium, 10% CS	1	Maintained five to seven days
5- 1-64	4	90% MEM, Eagle, 10% CS	10	Cells apparently healthy at two weeks, but no attachment

TABLE IV (Continued)

IN VITRO CULTURE OF EHRLICH ASCITES TUMOR CELLS

Date	No. of Cultures	Medium*	No. of Cells x 10^6 Bottle	Results
4-28-64	3	90% MEM, Eagle, 10% CS	2	Cloning and proliferation evident at three weeks; subcultured
4-28-64	3	90% LAH medium, 10% CS	2	Nil--less than three days
4-28-64	2	90% DYE medium, 10% CS	2	Nil--less than three days

*For media number designations, see text, Media, p. 22.

From the results shown in Table IV, there is no evidence that varying the number of initial cells or calf serum content either supports or hinders the growth of S-37 *in vitro*; not even a trend is observable.

The partial success of the S-37 cultures prompted the culture attempts with EAT listed at the ending of Table IV. Negative results for EAT with LAH and DYE media were observed, just as for S-37 with LAH medium. However, Eagle's medium was as beneficial for EAT as for the most successful S-37 cultures. The course and behavior of EAT was the same as for S-37 in almost every respect. One notable difference, however, was that EAT cells manifested both the round, epithelial-type cells and pointed, spindle-shaped, fibroblast-like cells. The EAT cells after subculture, however, were more uniformly round, and these were the cells which served in further *in vivo* work.

B. LD_{50} DETERMINATIONS

1. Mouse-passage Strains of EAT and S-37.

In order for measurements of the virulence of tissue culture cells to have meaningful interpretation, a determination of the quantitative virulence of the parent mouse-passage strain from which the tissue culture cells were derived was considered a necessary prerequisite. LD_{50} determinations served as the measuring devices for these comparative virulence studies.

Table V gives the results of LD_{50} determinations for the mouse-passage strains of S-37 and EAT. An LD_{50} determination for EAT iv was not run in this case since the former study (Oshiro, 1963) had shown that $>10^6$ cells were required. No reliable LD_{50} is attainable by this route as injection of cells in excess of this amount leads to vascular occlusion and mechanical damage to the mouse.

TABLE V

LD_{50} DETERMINATIONS FOR S-37 TUMOR CELLS

Dose	Mortality Ratios ip	sc	iv
10^2	--	--	0/7
10^3	2/10	--	2/9
10^4	1/9	3/9	4/9
10^5	6/9	5/9	5/5
10^6	--	10/10	--
LD_{50}*	57,700	66,700	8,992

LD_{50} DETERMINATIONS FOR EHRLICH ASCITES TUMOR CELLS

Dose	Mortality Ratios ip	sc	iv
10	3/10	--	--
10^2	7/9	--	--
10^3	9/9	1/9	--
10^4	--	7/9	--
10^5	--	10/10	--
LD_{50}*	48.7	6,130	--

*Computed by the method of Reed and Muench (Woolf, 1963)

The mortality ratios reflect deaths from tumor noted and recorded up to thirty days after injection. Animals dying from causes other than tumor were not included in totals. Mice injected ip showing obvious ascites at thirty days were included with deaths, as well-progressed tumors seldom, if ever, regress. Likewise, animals injected sc showing palpable subcutaneous tumors at thirty days were included with the deaths. Because injection of S-37 iv induces lung tumors, mice in these groups showing lung tumors at autopsy were grouped with the deaths also. These lung tumors ranged in size from pinpoint lesions to multiple large lesions, some of which adhered to the cell wall.

2. In Vitro Strain of EAT

Cells harvested from in vitro cultures of EAT after six weeks in culture were employed as a challenge inoculum. The cells were injected using the same experimental design as for the above mouse-passage strain of EAT, with the omission of 10^5 cells via the sc route. The data are shown in Table VI. Even the best culturing and harvesting procedures failed to yield sufficient numbers of cells for the complete study. Likewise, a sufficient number of S-37 cells was not obtainable for a parallel study.

A very definite trend is observable when the results shown in Table VI are compared with Table V. A decrease in virulence is apparent for both routes of injection. That is, more than 10^3 cells, rather than approximately 50 cells, are now required via the ip route to effect tumor formation and death of 50% of the animals. Likewise, greater than 10^4 cells compared to approximately 6,000 via the sc route are necessary to induce solid tumors.

TABLE VI

LD_{50} DETERMINATIONS FOR *IN VITRO* STRAIN OF EHRLICH ASCITES TUMOR CELLS

Dose	Mortality Ratios		
	ip	sc	iv
10	0/7	--	--
10^2	4/10	--	--
10^3	4/9	0/10	--
10^4	--	0/10	--
10^5	--	--	--
LD_{50}	$>10^3$	$>10^4$	--

C. IMMUNOGENICITY OF IN VITRO STRAIN OF EAT

If, in fact, the trend toward decreased virulence could be demonstrated to be real and readily repeated, the next logical step would be to determine the immunogenicity of such "weakened" or "attenuated" cells.

The poor yield of cells harvested from cultures curtailed investigation in this area. The final mortality ratio (8/9) would indicate a lack of protection. However, any definitive statement as to even a trend in this regard must await further experimentation.

D. REFRIGERATED S-37 TUMOR CELLS

As an alternate approach to the basic research problem, tumor cells which had been stored in the refrigerator for varying lengths of time were employed as another possible source of viable, attenuated, and potentially immunogenic cells. The experiments were designed to determine the extent to which such cells retain their original virulence, and whether or not they may be immunogenic.

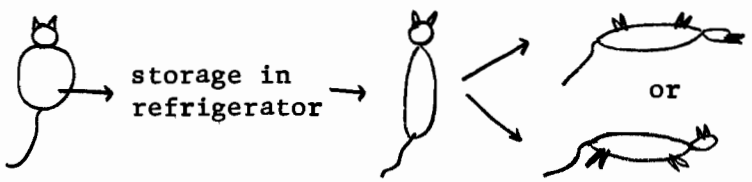
1. Retained Virulence

As will be noted from Table VII, per cent viability (as indicated by eosin dye exclusion) rapidly decreased upon refrigeration. Also, the data suggest a declining tumorigenicity of the cells upon prolonged refrigeration. However, the significance of these observations may be more apparent than real.

No statistically significant differences among the observed results were found when the data were analyzed by the Fisher non-parametric test (Finney, 1948). (Chi square analysis was not applicable to these data.) One possible contributing factor to this non-significant result may have been the unexplained behavior of the control group. Mice inoculated with freshly-drawn, unrefrigerated cells at this

TABLE VII

RETAINED VIRULENCE OF REFRIGERATED S-37 CELLS



Refrigeration Time	Per Cent Viable Cells	Mortality Ratio
15 weeks	0	0/4
5 weeks	0	0/9
1 week	8	3/7
0	84	1/9

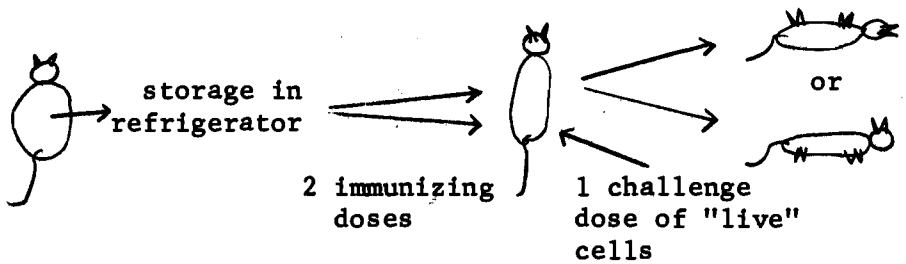
dosage would be expected to yield a 50% mortality ratio. The reasons for the observed (1/9) mortality ratio are not apparent. No conclusions may be drawn from the results of these studies.

2. Immunogenicity

An inspection of the mortality ratios listed in Table VIII reveals only inconsistent results. No trends are noticeable. Analysis by Chi square, applicable in a 2 x 2 contingency table, grouping the 3 "treated" groups versus the 2 "control" groups (Siegel, 1956) demonstrates no statistically significant differences; i.e., $p = 0.20$ to 0.30. The need for more definitive experiments is again evident.

TABLE VIII

IMMUNOGENICITY OF REFRIGERATED S-37 CELLS



Refrigeration Time	Mortality Ratio
15 weeks	5/9
5 weeks	9/9
1 week	2/5
0 - "immune" controls	3/9
0 - normal controls	6/10

V. DISCUSSION

A. IN VITRO CULTURES

An apparent anomaly exists between the many successful cultures and establishment of cell lines which have been cited and the essentially negative results of culture attempts reported in the preceding section. Reasons for the limited success in culturing tumor cells of different types described in this thesis are not readily evident.

A line of reasoning to explain limited success in cell culture begins with the premise that differences do exist among tumors, and even among strains of the same tumor, particularly with respect to nutritional requirements in culture. A medium which is nutritionally adequate for one tumor may be too refined for another or totally lacking in specific growth factors for yet another tumor or strain of the same tumor.

Evidence derived from the work with Cailleau and Costa's strain of EAT supports this latter hypothesis. Subcultures of this strain were readily passaged and maintained when the medium prescribed by Cailleau for this strain was utilized. Marked changes in composition of the medium failed to support further growth. Likewise, attempts to employ this medium for cultures of the one other strain of EAT investigated in these studies failed.

Hauschka and Levan (1958) emphasized another aspect of the problem of culturing tumor cells. These authors report "chromosome individuality" in studies with EAT and Krebs-2 ascites tumor reflected by variations in virulence, chromosome number, and other properties of clones of cells from the same neoplasm, even within the same strain.

By contrast with less successful efforts, such accomplishments as those of Foley et al. (1960) in establishing fifty-one new cell lines all

utilizing essentially the same medium, must be noted. Satisfying the individually fastidious cultural demands is apparently only part of the task in achieving successful cultures.

Reasons for discontinuing experiments with the tissue culture strain of EAT after successful subcultures for over one year were given in the preceding section. A word of further explanation is warranted. Since Cailleau and Costa had maintained this cell line for over two years prior to the time it was received in this laboratory, the total time in culture was over three years. Cailleau and Costa (1961) indicated that continued time in culture resulted in increasing loss of virulence. These observations, coupled with failure of mice to develop tumors when injected with cells from culture, indicated that the tissue culture cells had lost the demonstrable virulence capacities needed for further research along the guidelines which had been projected.

In other areas of inquiry, questions arise as to the identity of the cells being cultured upon primary isolation from the mouse. Whether the cells observed are actually tumor cells or possibly fibroblasts of the connective tissue incompletely removed from the tumor remains to be shown. Experimentation involving further inoculation of harvested cells into susceptible animals for tumor growth as well as histotogical studies would be desirable.

Another matter for consideration involves the implied requirement for attachment as a prerequisite for growth of EAT. The greatest yield of cells from the cultures reported in the previous section was from subcultures showing clusters of floating cells in increasing numbers. Viability and tumorigenicity of these cells were borne out in subsequent mouse reinoculation studies. Apparently a suspension culture had been effected

or at least cell maintenance was achieved, such that attachment in this case was not required.

B. LD_{50} DETERMINATIONS

The LD_{50} determinations for the mouse passage strains of S-37 and EAT showed good correlation with the former LD_{50}'s for the same tumors (Oshiro, 1963). The stability of the virulence characteristics of the tumor over long periods of extended serial passage in mice is thus established. Table IX lists these comparative values with 95% confidence limits for the former set shown in parenthesis.

On first impression it may appear from the new values that more cells are required via ip and iv routes for S-37 and via the ip route for EAT with fewer cells via sc routes for both tumors. However, a closer examination by statistical methods shows that no significant differences exist; this statement is documented in Figure I.

If the 95% confidence limits for two sets of values overlap, it is generally accepted that no significant differences exist between the results. Likewise, if an observed value falls within the specified limits, the confidence limits of the new observed value would obviously overlap the former limits. This second criterion is met in this instance for the four values where the 95% confidence limits are indicated. For EAT ip, confidence limits for the new value yields a range of 48.7 ± 53.4. Thus, the former value falls within these limits and hence, the difference here is likewise probably not significant.

Oshiro concluded from his LD_{50} values that variation in resistance to these tumors exists depending on the route of inoculation. This can be readily observed from Figure I to be the case for EAT. However, applying the 95% confidence limits rule to S-37 shows that there are no statistically significant differences among the three routes. Nevertheless,

TABLE IX

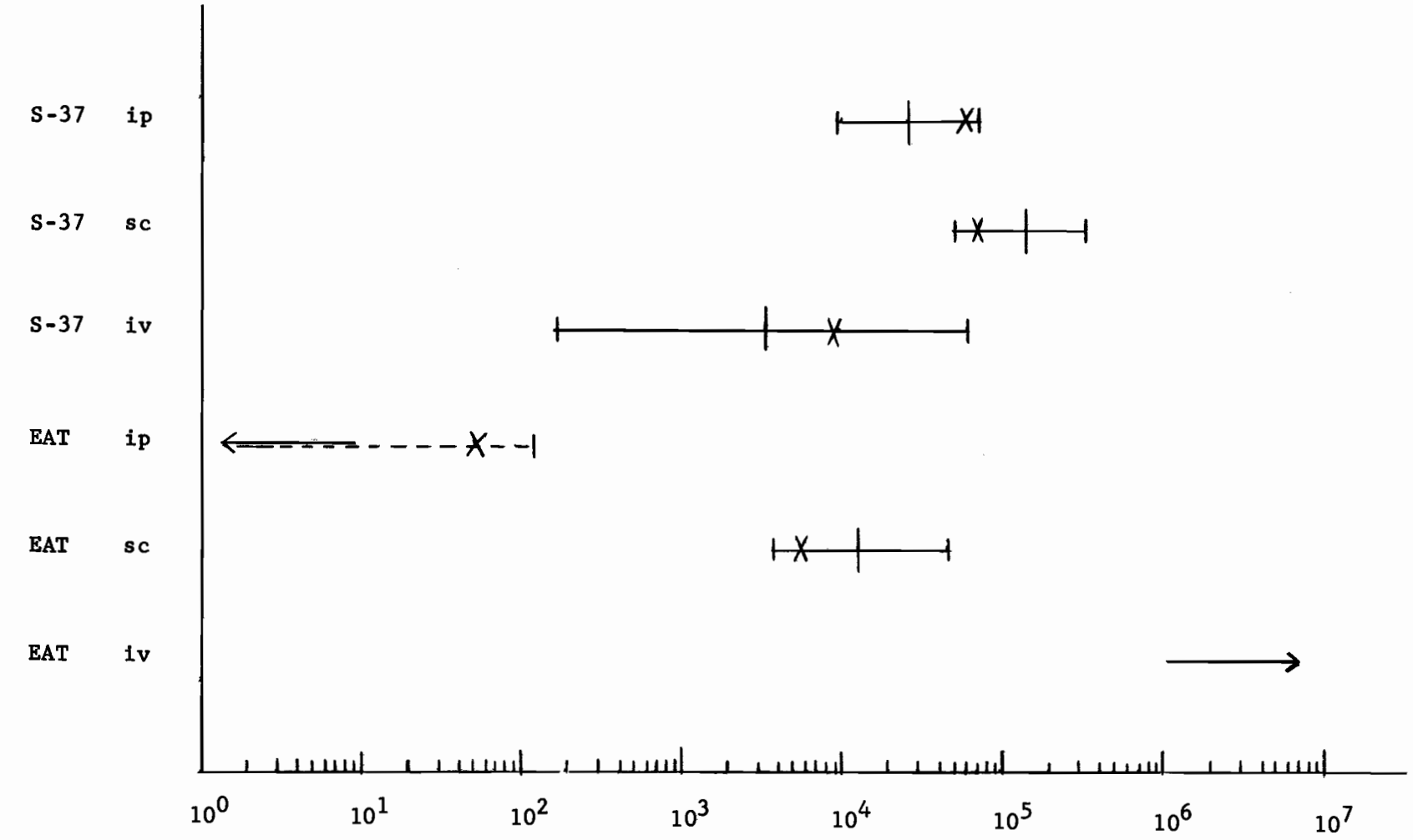
COMPARISON OF LD_{50} DETERMINATIONS

Tumor	Route	Present LD_{50}	Former LD_{50} (95% confidence limits)
S-37	ip	57,700	25,000 (8,928 - 70,000)
S-37	sc	66,700	125,000 (52,083 - 300,000)
S-37	iv	8,992	3,200 (160 - 64,000)
EAT	ip	48.7	< 10
EAT	sc	6,130	13,000 (3,714 - 45,500)
EAT	iv	--	$> 10^6$

FIGURE I

COMPARISON OF LD_{50} DETERMINATIONS FOR

S-37 AND EHRLICH ASCITES TUMOR CELLS



observing the mortality and responses of the animals tends to lend credence to the concept that these differences are real even though the actual values are not significantly different by conventional interpretation.

Using the same strains of S-37 and EAT as those used in these experiments, Bringhurst (1964, personal communication) found that at least 10^3 EAT cells given ip were required as an LD_{50} for mice. However, at least two other investigators working with other strains of EAT (Cailleau and Costa 1961; and Lindenmann, 1964) have reported that approximately ten cells injected ip will induce tumors in 50% of the test animals. These latter reports tend to verify the findings presented in the previous section.

Sources of error inherent in the procedures for determining LD_{50} values include the following: a) condition and age of cells when withdrawn from the mouse, i.e. young, dividing, mature, degenerating, etc.; b) viability as reflected by eosin-staining; c) arithmetic manipulations for counting and diluting; d) diluting technique; and e) variation in individual mouse susceptibilities. Each of these factors may contribute to the causes of the discrepancies noted above.

The decreased virulence noted in the LD_{50} determination for EAT after six weeks in culture appears to be both statistically and empirically significant. The scope of the experiment should be enlarged to involve more animals, more doses, and more observations from different lengths of time in culture before a definite statement can be made.

C. IMMUNOGENICITY OF *IN VITRO* EAT

Further work would be desirable to study the possible immunogenicity of cells from *in vitro* cultures. The limited number of cells available, the small number of mice inoculated, the inadequate immunizing doses and excessive challenge dose all combined to make the reported experiment a relatively unsatisfactory one.

D. REFRIGERATED S-37 CELLS

The inconsistent and inconclusive results from the refrigerated cell experiments with little if any predictable behavior being borne out even by the control groups may have been due to several factors. First, the number of cells in the dose for retained virulence, 10^4, was below the LD_{50}. However, this dose is still within the range that tumors should occur in about one-third of the mice. Second, if the stained cells were in fact non-viable, the total number of viable cells actually injected was proportionately less than 10^4 cells. A third factor was that the mice used were all young, female mice; these latter two characteristics might contribute to more resistance, since older mice are more susceptible than young mice and females are more resistant than males. On the basis of these observations, further experiments should utilize a range of 10^4 and higher doses, and groups of older mice of both sexes.

VI. SUMMARY

Initial efforts to achieve successful in vitro culture of EAT cells using several media under a variety of conditions yielded only limited success. Subcultures of another, tissue culture strain of EAT were maintained successfully for over one year. Culture attempts with solid tumors of inbred strains of mice gave few positive results. S-37 ascites tumor cells and subsequently the original EAT strain were passaged through subculture for over six weeks with sufficient cells being harvested for succeeding mouse reinoculation studies on a pilot basis.

Determinations of LD_{50} values for mouse-passage strains of EAT and S-37 revealed good correlation with former values, indicating stability of the virulence characteristics of the tumor cells upon continued mouse passage. LD_{50} determinations for EAT cells after six weeks in culture demonstrated an apparent decrease in the virulence of this in vitro strain in comparison to the observed LD_{50} values of the mouse passage strain. An insufficient yield of cells from further subcultures prevented the continuation of comparative virulence studies for both EAT and S-37. Immunogenicity of these cells, likewise, could not be demonstrated under these conditions.

No discernible trends could be observed in the results of the experiments designed to test for retained virulence and immunogenicity of the refrigerated S-37 tumor cells. Comparisons of the mortality ratios for mice treated with refrigerated cells and those of immune and normal controls exhibited no statistically significant differences.

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